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A novel wheat gene encoding a putative chitin-binding lectin is associated with resistance against Hessian fly

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SUMMARY

The gene-for-gene interaction triggering resistance of wheat against first-instar Hessian fly larvae utilizes specialized defence response genes not previously identified in other interactions with pests or pathogens. We characterized the expression of *Hfr-3*, a novel gene encoding a lectin-like protein with 68–70% identity to the wheat germ agglutinins. Within each of the four predicted chitin-binding hevein domains, the HFR-3 translated protein sequence contained five conserved saccharide-binding amino acids. Quantification of *Hfr-3* mRNA levels confirmed a rapid response and gradual increase, up to 3000-fold above the uninfested control in the incompatible interaction 3 days after egg hatch. *Hfr-3* mRNA abundance was influenced by the number of larvae per plant, suggesting that resistance is localized rather than systemic. In addition, *Hfr-3* was responsive to another sucking insect, the bird cherry-oat aphid, but not to fall armyworm attack, wounding or exogenous application of methyl jasmonate, salicylic acid or abscisic acid. Western blot analysis demonstrated that HFR-3 protein increased in parallel to mRNA levels in crown tissues during incompatible interactions. HFR-3 protein was detected in both virulent and avirulent larvae, indicating ingestion. Anti-nutritional proteins, such as lectins, may be responsible for the apparent starvation of avirulent first-instar Hessian fly larvae during the initial few days of incompatible interactions with resistant wheat plants.

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INTRODUCTION

Plant tissues contain several types of carbohydrate-binding proteins designated as lectins or agglutinins. These proteins cause agglutination of cells and/or precipitation of glycoconjugates. Seven families comprise this class of protein domains (reviewed in Van Damme *et al.*, 1998), which includes the legume lectins, type-2 ribosome-inactivating proteins, the amaranthin family, the Cucurbitaceae phloem lectins, monocot mannose-binding lectins, jacalin-like lectins and hevein chitin-binding lectins.

Of greatest interest in this study are lectins that may play a role in the resistance of wheat, *Triticum aestivum* (L. em Thell), against insects. The amaranthin lectins are seed proteins presumed to be associated with defence against seed predators. However, we identified the *Hfr-2* gene, a Hessian fly (*Mayetiola destructor* [Say])-responsive wheat gene containing an amaranthin lectin domain, which is expressed in leaf sheathes at the crown of the plant and minimally in the seeds during compatible interactions (Puthoff *et al.*, 2005). The monocot mannose-binding lectins (reviewed in Van Damme *et al.*, 1998) serve as storage proteins and may act against sucking insects as this class of lectins accumulates in the phloem sap. These lectins bind to mannose-type glycan chains, which are typical constituents of insect glycoproteins in the midgut. Jacalin-like lectins have also been shown to have anti-insect properties. Previous work in our laboratory demonstrated that wheat *Hfr-1* and *Wci-1* mRNAs, encoding jacalin-like mannose-binding lectins, become more abundant in response to avirulent Hessian fly larval feeding. The *Hfr-1* gene is proposed to be involved in the defence response against this insect (Subramanyam *et al.*, 2006; Williams *et al.*, 2002).

Another class of lectins, which includes wheat germ agglutinins (WGAs), reversibly binds to chitin, a β-1,4-linked biopolymer of *N*-acetylglucosamine (GlcNAc), as well as to glycoconjugates that contain GlcNAc or *N*-acetylneuraminic acid (NeuNAc; Raikhel *et al.*, 1993). These chitin-binding lectins with hevein domains are associated with plant defence against insects and may bind to endogenous GlcNAc for storage of these oligomers as signalling molecules, or to exogenous GlcNAc-containing

signalling molecules to prevent stimulation of plant responses. Proteins with chitin-binding properties, such as the chitin-binding lectins, have a common structural motif of 30–43 amino acids containing conserved cysteine and glycine residues (Raikhel *et al.*, 1993) and have a negative effect on the growth and development of chitin-containing organisms such as insects and fungi (Chrispeels and Raikhel, 1991). A potential target for chitin-binding lectins in insects is the peritrophic matrix (PM), a midgut-secreted envelope composed of chitin, proteins, glycoproteins and glycosaminoglycans (Murdock and Shade, 2002). The PM of phytophagous insects, such as the Hessian fly, is directly exposed to food and other ingested chemicals derived from the host plant. Because the insect PM contains glycoproteins and chitin, it harbours many potential binding sites for dietary lectins such as chitin-binding proteins (Peumans and Van Damme, 1995).

WGA is one of the best-characterized chitin-binding lectins and consists of three isoforms, A and D (Smith and Raikhel, 1989) and B (Raikhel and Wilkins, 1987). Artificially feeding WGA to cowpea weevil larvae, *Callosobruchus maculatus* (L.), delayed larval development (Murdock *et al.*, 1990). The Australian sheep blowfly, *Lucilia cuprina* (Wiedemann), a dipteran like the Hessian fly, showed inhibited larval growth and increased larval mortality when fed WGA (Eisemann *et al.*, 1994). Feeding WGA caused reduced larval weight and increased mortality in the European corn borer, *Ostrinia nubilalis* (Hübner), and the Southern corn rootworm, *Diabrotica undecimpunctata howardi* (Barber), both of which are important pests of corn (Czapla and Lang, 1990). WGA-fed *O. nubialis* developed abnormalities in the PM structure including large holes in the chitin meshwork of the envelope and disruption of the microvillar organization in the midgut (Harper *et al.*, 1998). The effects of WGA on insect development suggest that this chitin-binding lectin may function as a feeding deterrent. Disruption of midgut function or repulsion due to WGA detection by insect gustatory receptors may greatly limit nutrient consumption, resulting in death by starvation.

Characteristics of the incompatible interaction between resistant wheat and avirulent first-instar Hessian fly larvae are suggestive of death by starvation. The larvae are unable to establish feeding sites among the leaf sheaths at the crown of the seedling and die within 3–4 days (Gallun, 1977; Grover, 1995). Because larval death is not immediate, starvation rather than acute toxicity appears to be the mode of death. Supporting this hypothesis is the observation that avirulent larvae do not increase in size, suggesting that they are unable to take in nutrients. Avirulent larvae do probe the plant, however, and a radioactive tracer assay demonstrated that they ingest a small amount of material while attempting to feed (Shukle *et al.*, 1990), but are unable to alter the physiology of the host.

Genetic characterization of wheat–Hessian fly interactions demonstrated that resistance is triggered by a resistance gene-mediated recognition of a larval gene-encoded avirulence factor (Flor, 1955; Gallun, 1978; Hatchett and Gallun, 1970). Although wheat–Hessian fly interactions are similar to plant–pathogen interactions in that they operate in this gene-for-gene manner, recent studies in our laboratory suggest that several mechanisms commonly associated with plant defence against microbial pathogens are not involved. First, no evidence of a classical oxidative burst was detected in wheat following attack by virulent or avirulent Hessian fly larvae (Giovanini *et al.*, 2006). Second, mRNA expression profiles of ten plant pathogenesis-related (PR) genes were examined in Hessian fly challenged wheat and all except *Wci-2* (lipoxygenase) were found to be minimally responsive to Hessian fly attack (Sardesai *et al.*, 2005).

Despite the fact that the phenotypic characteristics (Cartwright *et al.*, 1959) and Mendelian genetics (Gallun, 1977; Hatchett and Gallun, 1970) of wheat–Hessian fly interactions have been described for some time, events at the molecular level contributing to susceptibility (Puthoff *et al.*, 2005) and resistance (Sardesai *et al.*, 2005) are only now being elucidated. *Hfr-1* and *Wci-1*, closely related wheat genes encoding jacalin-like mannose-binding lectins, respond to avirulent Hessian fly larval feeding with increased mRNA abundance at the feeding site (Subramanyam *et al.*, 2006; Williams *et al.*, 2002). But the two genes differ in their responsiveness to other biotic (aphid/virus infection) or abiotic (mechanical wounding) factors, with *Hfr-1* being selectively responsive to Hessian fly larvae. The resistance mechanisms comprise a network of inducible responses (C. E. Williams *et al.*, in preparation) believed to be initiated by *R* gene-mediated recognition of larval salivary elicitors delivered to the plant during early stages of the interaction.

The work described here was carried out in order to understand better the mechanisms of wheat resistance to Hessian fly attack. In this report, we present the characterization of *Hfr-3* (Hessian fly responsive-3), a novel wheat lectin-like gene that encodes a protein containing four chitin-binding domains, similar to WGA. At the crown of the plant where first-instar larvae attempt to feed, *Hfr-3* mRNA and HFR-3 protein levels increased over the first 4 days of incompatible interactions. In addition, HFR-3 protein was detected in the body of Hessian fly larvae, suggesting a role in defence against this insect. Indeed, the presence of chitin-binding domains in the HFR-3 deduced amino acid sequence suggests that HFR-3 targets the PM of the larval midgut and contributes to avirulent larval mortality.

RESULTS

Hfr-3 cloning and sequence annotation

An expression profiling technique (GeneCalling; Curagen Corp., New Haven, CT) identified a wheat sequence responsive to attack by avirulent Hessian fly larvae (incompatible interaction). A near full-length cDNA clone (UPW1Hfr3) corresponding to this wheat

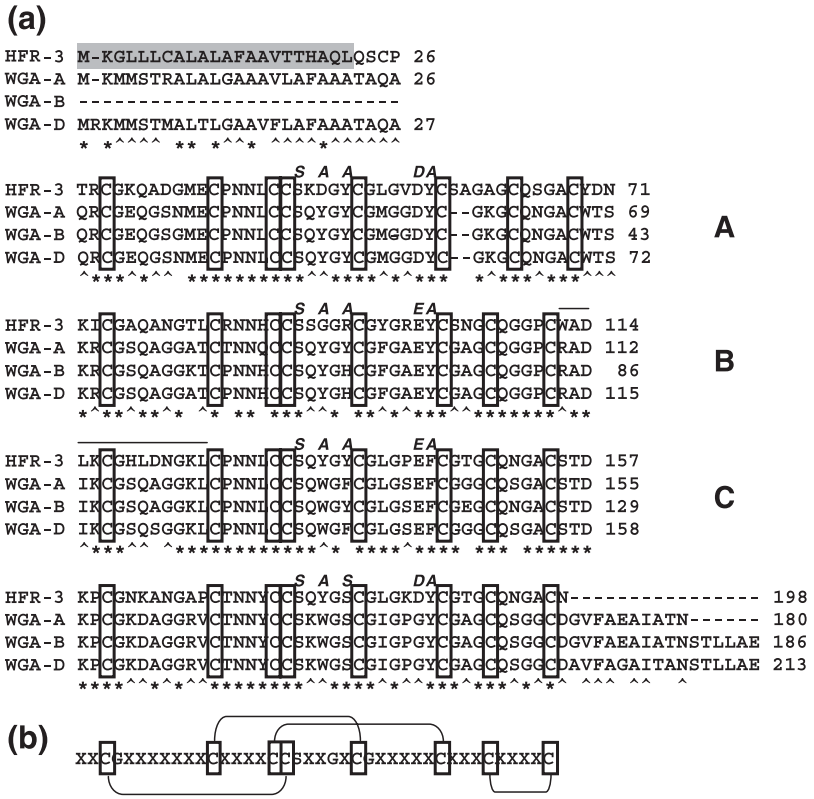

Fig. 1 HFR-3 deduced amino acid sequence. (a) HFR-3 sequence aligned with WGA sequences. An asterisk indicates that the amino acids in that alignment column are identical in all sequences having an amino acid at that position. A circumflex (^) indicates that the HFR-3 sequence differs from all WGAs that have an amino acid at that position. Letters in italics above the sequence refer to saccharide-binding residues conserved in hevein domains. *S* refers to serine. *A* refers to aromatic amino acids: phenylalanine (F), tyrosine (Y) or tryptophan (W). *D* and *E* refer to aspartate and glutamate, respectively. The four putative chitin-binding type-1 hevein domains (A–D) each contain eight conserved cysteine residues (boxed). The grey shaded sequence shows the predicted signal peptide for HFR-3. The line above amino acids 112–125 in the HFR-3 sequence indicates the sequence of the synthetic peptide that was used to raise the HFR-3 antibody. (b) Chitin-binding type-1 hevein domain consensus motif. Each domain features a common structure consisting of 30–43 residues organized around a conserved four-disulphide core (disulphide bonds indicated with brackets), as described in Raikhel *et al.* (1993).

sequence was obtained by RACE PCR and the gene was named *Hfr-3*. The *Hfr-3* cDNA contains a 594-bp open reading frame (ORF) sequence (Accession no. DQ462308) that encodes a deduced protein sequence of 198 amino acids (Accession no. ABE77384).

Structural analysis of *Hfr-3* revealed that the gene has no introns; primers designed from the *Hfr-3* cDNA sequence to amplify the entire coding region of *Hfr-3* amplified a fragment from the genomic DNA identical to the cDNA sequence of *Hfr-3*. The *Hfr-3* ORF shares 54% identity with wheat germ agglutinin-A (WGA-A; *e*-value 7e-11; Accession no. M25536), 55% with WGA-B (*e*-value 3e-16; Accession no. J02961) and 55% with WGA-D (*e*-value 1e-15; Accession no. M25537). Using the BLASTp sequence similarity search tool (Altschul *et al.*, 1997), the HFR-3 deduced amino acid sequence was found to be 68% identical to WGA-A (*e*-value 6e-67; Accession no. P10968), 70% identical to WGA-B (*e*-value 2e-69; Accession no. P10969) and 69% identical to WGA-D (*e*-value 5e-68; Accession no. P02876). Alignment of the WGA and HFR-3 translated protein sequences revealed conservation of residues throughout the ORFs (Fig. 1a).

The program ScanProsite (Gattiker *et al.*, 2002) identified four chitin-binding type-1 hevein domains in the deduced HFR-3 amino acid sequence (denoted A–D in Fig. 1a). The cysteine residues form a four-disulphide core within the common structural motif of each chitin-binding domain (Fig. 1b). The positions of these cysteine residues are conserved in the WGA-A, -B and -D proteins (Fig. 1a) and the disulphide bonds maintain the three-dimensional shape of the WGA proteins.

Each consensus chitin-binding hevein domain includes five conserved saccharide-binding amino acids (Fig. 1a): aromatic amino acids at positions 21, 23 and 30 (*A*), a serine at position 19 (*S*) and an aspartate or glutamate at position 29 (*D* and *E*). However, position 23 is more variable, lacking an aromatic amino acid in some of the domains of the three WGA proteins as well as in the second and fourth domains of HFR-3. In addition, HFR-3 lacked aromatic amino acids at position 21 of the first two chitin-binding domains. All WGA and HFR-3 chitin-binding domains contained aromatic amino acids at the remaining consensus positions in addition to the conserved serine at position 19. Also, the glutamate or aspartate at position 29 was conserved in all HFR-3 domains, but is lacking in the fourth domain of the three WGA proteins (Fig. 1a).

Like the WGA proteins, the deduced HFR-3 protein sequence contained an N-terminal putative secretory signal peptide sequence and a section with four chitin-binding motifs (Fig. 1a). However, the *Hfr-3* termination codon occurred only three bases after the end of the fourth chitin-binding domain-encoding sequence. Consequently, the HFR-3 sequence was lacking a C-terminal propeptide (15 amino acids) that targets the WGA proteins to vacuoles.

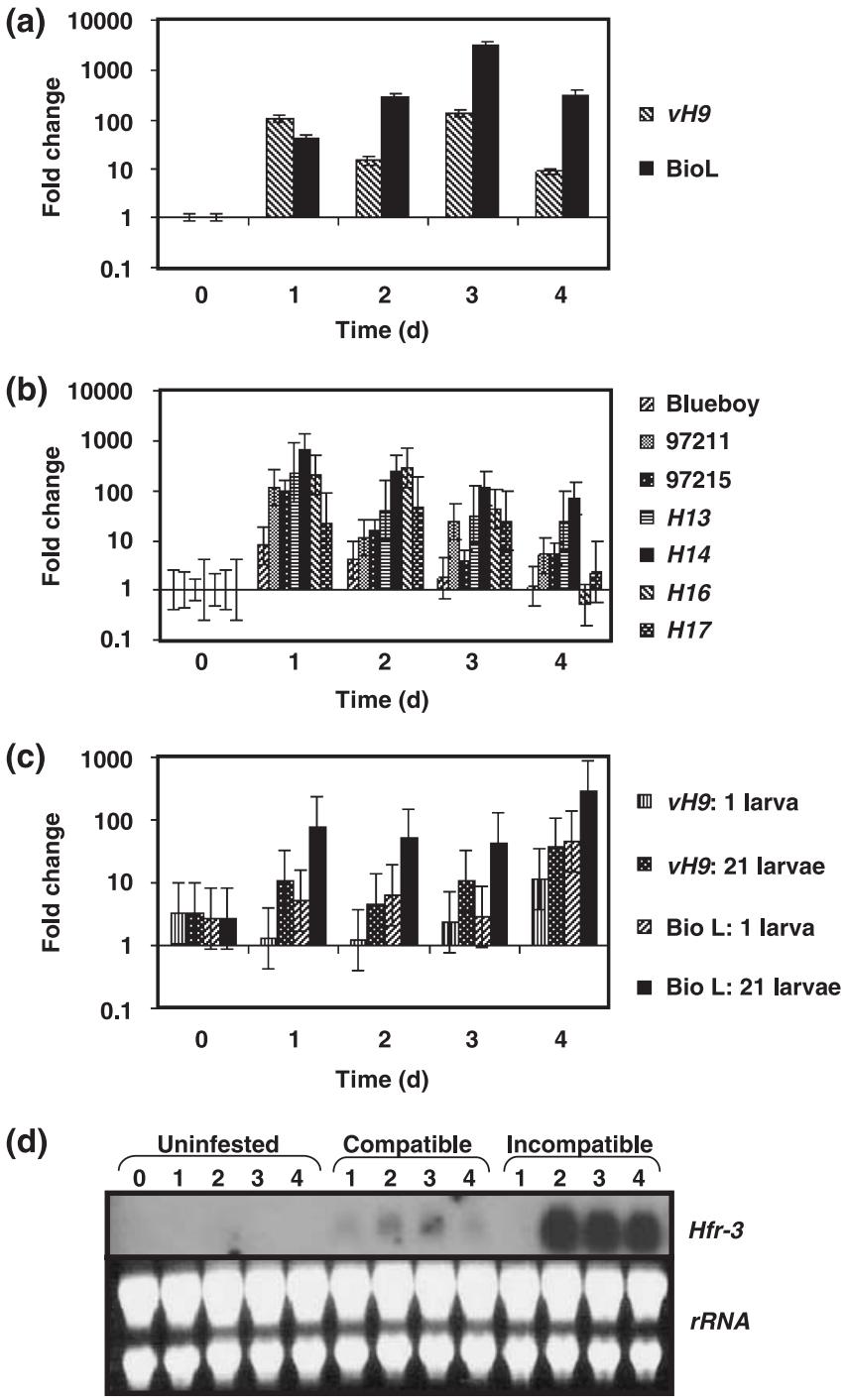

Fig. 2 *Hfr-3* transcript levels. Crown tissues were collected 2 days after infestation, which is 2 days prior to egg hatch (time 0), and 1–4 days after egg hatch. qRT-PCR analysis utilized the standard curve method to determine mRNA levels. Fold-change above uninfested controls and standard error (a–c) were calculated from the ANOVA test. Each graph represents results from two independent experiments, and all samples were subjected twice to qRT-PCR. The baseline (fold change of 1) corresponds to the *Hfr-3* mRNA levels in uninfested control plants. (a) *Hfr-3* mRNA levels in *H9*-Iris plants during compatible interactions (infested with *vH9* Hessian flies) and incompatible interactions (infested with Biotype L). (b) *Hfr-3* mRNA levels in six different resistant wheat lines (each line contains a different Hessian fly resistance gene) infested with Biotype L Hessian fly to produce incompatible interactions and one susceptible wheat line, Blueboy, infested with Hessian fly Biotype L to produce a compatible interaction. (c) *Hfr-3* transcript levels in *H9*-Iris plants infested with one vs. 21–30 Hessian fly larvae per plant. (d) Top: autoradiogram of a Northern blot of total RNA extracted from *H9*-Iris crown tissues of uninfested plants and plants involved in a compatible interaction (infested with *vH9* Hessian flies), and incompatible interaction (Hessian fly Biotype L), on days 0–4 (as above). Total RNA was probed with a ^{32}P-labelled *Hfr*-3 clone (UPW1Hfr3) and exposed to film for 5 days. The ethidium bromide-stained rRNA is shown to demonstrate equal loading of the total RNA.

Hfr-3 expression in response to Hessian fly larval attack

qRT-PCR was used to determine *Hfr-3* mRNA levels in the crown tissues of plants during the first 4 days of compatible (*H9*-'Iris' wheat with *vH9* larvae) and incompatible (*H9*-Iris wheat with Biotype L larvae) interactions (Fig. 2a). At most time points, *Hfr-3* mRNA was more abundant in incompatible than in either compatible interactions or in uninfested plants. *Hfr-3* mRNA levels were highest in the incompatible interaction 3 days after egg hatch (3000-fold increase above uninfested control; $P < 0.0001$). qRT-PCR was also carried out with primers designed to amplify a conserved region from *WGA-A, -B* and *-D* using the same samples that showed differential levels of *Hfr-3* message. Although low

Table 1 Peak fold-change of *Hfr-3* transcripts in wheat lines infested with Biotype L Hessian fly larvae.

Wheat line (resistance gene)	Peak fold-change above uninfested control	*P*-value	Day(s) after egg hatch
Blueboy no resistance	7.8	0.0559	1
97211 unnamed gene	118*	0.0005	1
97215 unnamed gene	96*	< 0.0001	1
Iris *H9*	3000*	< 0.0001	3
Molly *H13*	235*	0.0004	1
921676 *H14*	645*	< 0.0001	1
921682 *H16*	281*	0.0003	2
921680 *H17*	47*	0.0245	2

*Significant fold change ($P < 0.05$).

levels of *WGA* mRNA were detected in all samples, the WGA genes were unresponsive to Hessian fly larvae (data not shown).

In order to determine whether increased *Hfr-3* mRNA abundance is a common response of wheat during incompatible interactions with avirulent Hessian fly larvae, *Hfr-3* mRNA levels were measured in additional wheat genotypes. Six wheat lines, all resistant to Biotype L Hessian fly larvae but each carrying a different Hessian fly resistance gene, plus susceptible wheat cultivar 'Blueboy' (lacks any Hessian fly resistance gene), were challenged with Biotype L. All wheat lines carrying a Hessian fly resistance gene responded with significant increases in *Hfr-3* mRNA levels (Fig. 2b, Table 1).

To investigate whether *Hfr-3* mRNA levels are influenced by the number of larvae feeding on a wheat seedling, *Hfr-3* transcript abundance was measured in crown tissues of plants containing one vs. a moderate infestation level of 21–30 Hessian fly larvae per plant. The highest *Hfr-3* mRNA levels occurred 4 days after egg hatch in this experiment (Fig. 2c). At this time point, all infested plants had significantly higher *Hfr-3* mRNA levels than uninfested control plants ($P < 0.05$) regardless of whether infested with one or 21–30 larvae. The trend at all post-hatch time points, in both compatible (*vH9* larvae on *H9*-Iris plants) and incompatible (Biotype L larvae on *H9*-Iris plants) interactions, was for plants with the moderate infestation level to have three- to 16-fold more *Hfr-3* mRNA than their counterparts infested with single larvae. This comparison was significant only in the incompatible interaction at 1 day (14.92-fold; $P = 0.0229$) and 3 days (15.87-fold; $P = 0.0205$) after egg hatch.

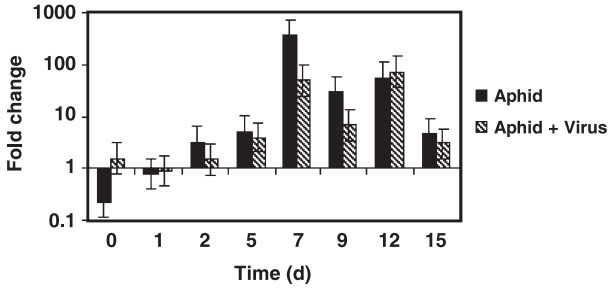

Fig. 3 *Hfr-3* response to aphid and virus. mRNA levels of *H9*-Iris wheat were quantified by qRT-PCR 1 day before infestation (time 0) and on indicated days during compatible interactions with bird cherry-oat aphid carrying no virus or aphid carrying both Cereal Yellow Dwarf and Barley Yellow Dwarf Viruses. Experimental design and data analyses were as described in Fig. 2.

Northern hybridization analysis was used to examine *Hfr-3*-like transcript accumulation from related genes. Because the qRT-PCR was specific to only the *Hfr-3* sequence, Northern hybridizations with near full-length cDNA clone UPW1Hfr3 would pick up transcripts from family members and would not match the qRT-PCR results if other copies of the gene were behaving differently. However, similar results to the qRT-PCR shown in Fig. 2a were observed. By Northern analysis, *Hfr-3*-like transcript levels were highest in the incompatible interaction at 2, 3 and 4 days after hatch of Hessian fly eggs (Fig. 2d). *Hfr-3*-like mRNA appeared to be moderately abundant as dark bands on the autoradiogram were visible after only 5 days of exposure.

Hfr-3 response to biotic and abiotic stresses

The response of *Hfr-3* to various biotic and abiotic stresses was assessed to determine whether the gene is part of a generalized stress response or is more specialized. *Hfr-3* mRNA levels were examined during compatible interactions following infestation of wheat seedlings with viruliferous bird cherry-oat aphids, *Rhopalosiphum padi* (L.), carrying both Cereal Yellow Dwarf Virus (CYDV) and Barley Yellow Dwarf Virus (BYDV), or non-viruliferous *R. padi* carrying no virus. No incompatible combination was available. Compared with uninfested controls, plants infested with non-viruliferous *R. padi* (compatible interaction) showed the most significant increase in *Hfr-3* mRNA 7 days after infestation (375-fold above uninfested; $P < 0.0001$; Fig. 3). The most significant increase in *Hfr-3* mRNA following viruliferous aphid infestation occurred 12 days after infestation (73.5-fold above uninfested; $P < 0.0001$; Fig. 3a). Viruliferous aphids resulted in a lower accumulation of *Hfr-3* mRNA compared with plants infested with non-viruliferous aphids, indicating a small negative effect of the virus in the accumulation of *Hfr-3* transcript.

Other stresses were applied to determine whether *Hfr-3* is part of a general stress response or is more specific to insect feeding. To determine whether *Hfr-3* message increased in abundance

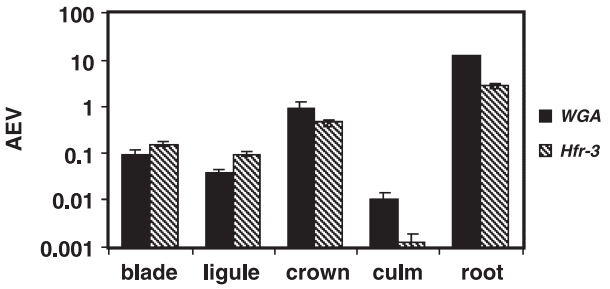

Fig. 4 *Hfr-3* mRNA levels in uninfested plant tissues. mRNA levels in different tissues of *H9*-Iris wheat were quantified by qRT-PCR. Culm tissue was from fully headed plants and other tissues were from two-leaf-stage seedlings. The primers for WGA were designed to amplify from *WGA-A*, *-B* and *-D* sequences. Arbitrary expression values (AEVs) were used as no comparison was made to infested tissue. Standard error was calculated from the ANOVA test.

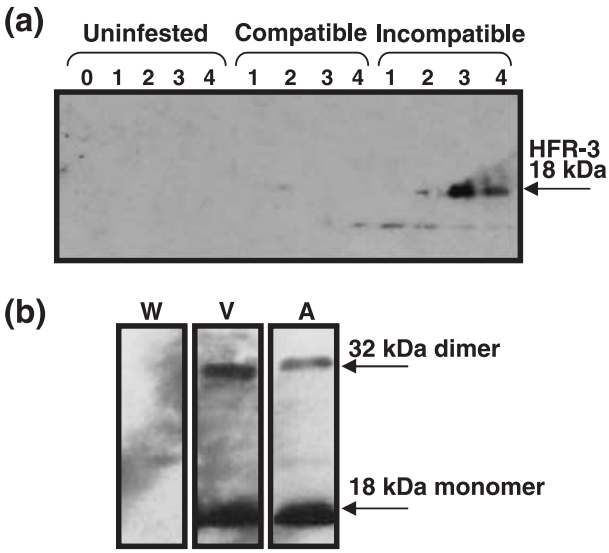

Fig. 5 Western blot analysis of HFR-3 protein from wheat and larval tissues. (a) *H9*-Iris wheat was infested with *vH9* (compatible interaction) and Biotype L (incompatible interaction) Hessian fly larvae. Infested crown tissues were collected 1–4 days after hatch of the eggs. Crown tissues were collected from uninfested control plants 2 days before (time 0) to 4 days after eggs hatched on infested plants. Immunodetection was carried out using an HFR-3-specific antibody raised against a synthetic peptide containing 14 residues from the HFR-3 deduced protein sequence. (b) Immunodetection of HFR-3 in the total protein of 12-h-old *vH9* larvae emerged in water (W), and in washed 3-day-old virulent *vH9* (V) and avirulent Biotype L (L) larvae raised on *H9*-Iris wheat using the same antibody as in (a).

following attack by a chewing insect whose feeding activity causes considerable mechanical damage, *Hfr-3* mRNA levels were measured in wheat seedlings infested with the fall armyworm (FAW), *Spodoptera frugiperda* (Smith). No significant change in *Hfr-3* mRNA level was detected during this compatible interaction at any time point (data not shown). In addition, no significant change in *Hfr-3* mRNA level was detected following mechanical wounding with forceps (data not shown). *Hfr-3* was not responsive to exogenous application of the resistance-signalling molecules abscisic acid (ABA) or methyl jasmonate (MeJA), and mRNA levels responded to salicylic acid (SA) only 3 days after treatment (47-fold increase above untreated; $P = 0.062$).

Hfr-3 and *WGA* tissue-specific mRNA levels

Hfr-3 and *WGA* transcript levels were analysed in different organs of uninfested wheat plants to determine whether expression of the two genes is similar in healthy plants even though expression differs in Hessian-fly-infested plants. *WGA* and *Hfr-3* mRNA levels were similar in all organs examined, except for culm (stem of mature plants) where *WGA* mRNA was more abundant than *Hfr-3* mRNA (Fig. 4). Even with this difference, the rank order for mRNA abundance in the various organs was the same for both genes.

Immunodetection of the HFR-3 protein

Western analysis was carried out to determine whether the increase in *Hfr-3* mRNA leads to more abundant HFR-3 protein in wheat tissues during incompatible interactions and to determine whether HFR-3 protein is present in the larval body due to ingestion. The HFR-3 antibody was raised against a synthetic peptide from amino acids 112–125 (Fig. 1a). The predicted molecular weight of the HFR-3 protein was determined from the deduced amino acid sequence to be 20.23 kDa. However, the mature protein detected on Western blots lacked the signal peptide (MKGLLLCALALAFAAVTTHAQL; Fig. 1a) and consequently was visualized as an 18-kDa protein (expected size of the monomer) in the crown tissues of plants during incompatible interactions (Fig. 5a). HFR-3 protein was not detected in the crown tissues of the control (uninfested) or plants involved in a compatible interaction. HFR-3 signal intensity in incompatible interactions reached its highest-level 3 days after egg hatch (Fig. 5a), in a profile similar to that observed for *Hfr-3* gene transcript accumulation (Fig. 2a).

Unlike the qRT-PCR experiments, the Western analysis did not yield quantitative data. We were compelled to attempt an estimation of the difference in HFR-3 protein abundance between compatible interactions (where the protein abundance is below detectable levels) and incompatible interactions (where the protein is visualized as a dark band), so a dilution experiment was performed. We determined that a sample yielding an HFR-3 band intensity similar to that seen in the incompatible interaction 3 days after egg hatch (Fig. 5a) could be diluted 30-fold before the protein was no longer detectable by the HFR-3 antibody under the conditions of our Western analysis. This experiment gave a conservative estimate that the protein is at least 30-fold

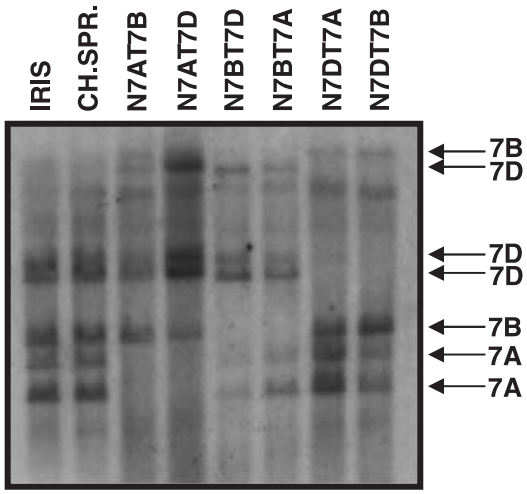

Fig. 6 *Hfr-3*-like sequences on homeologous group 7 chromosomes of wheat. An autoradiogram of a Southern blot shows hybridization of a ^{32}P-labelled *Hfr-3* clone (UPW1Hfr3) with genomic DNA from group 7 nullitetrasomic Chinese Spring-derived wheat stocks and *H9*-Iris wheat. DNA was digested with *Eco*RV endonuclease, which does not cut within the coding sequence. Lanes are labelled to represent each wheat line (example: N7AT7B is missing both copies of chromosome 7A, has four copies of 7B and two copies of 7D). Arrows indicate bands missing in the nullitetrasomic lines when compared with Chinese Spring and *H9*-Iris genomic DNA.

more abundant in the incompatible than in the compatible interaction or controls (data not shown). Owing to low conservation within the target sequence (eight of 14 amino acids were identical), HFR-3 antibody did not cross-react with commercially available WGA protein during Western blot analysis (data not shown), whereas purchased WGA antibody verified that the commercially available WGA protein was not degraded.

HFR-3 protein was also detected in the total protein extract from washed virulent (*vH9*) and avirulent (Biotype L) larvae (Fig. 5b), indicating ingestion of HFR-3 by the larvae during attack. The HFR-3 antibody did not detect protein in 12-h-old virulent or avirulent larvae that were emerged in water and had never fed on wheat plants. The same results were seen on Western blots in two additional biological replicates.

Hfr-3 genomic location

Southern hybridization experiments using genomic DNA from nullitetrasomic wheat stocks and ^{32}P-labelled *Hfr-3* probe UPW1Hfr3 were conducted to determine the chromosomal locations of *Hfr-3*-like sequences in the hexaploid wheat genome. Southern analysis using 21 nullitetrasomic lines indicated that *Hfr-3*-like sequences are located on the homeologous group 7 chromosomes (7A, 7B and 7D; data not shown). To confirm this result, genomic DNA samples from the six nullitetrasomic lines for group 7 were digested with *Eco*RV (does not cut within the coding sequence of *Hfr-3*) and probed, revealing that multiple *Hfr-3*-like sequences are present on each of the homeologous group 7 chromosomes of the hexaploid wheat genome (Fig. 6). No cross-hybridization was seen between the *Hfr-3* probe and *WGA* sequences, which reside on the group 1 chromosomes.

DISCUSSION

The experiments described here, focusing on the *Hfr-3* gene, are part of a larger project to characterize the molecular mechanisms of wheat resistance against Hessian fly attack. Our previous work demonstrates that mRNAs from *Hfr-1* and *Wci-1*, both encoding wheat jacalin-like mannose-binding lectins, become more abundant in response to probing by avirulent first-instar Hessian fly larvae (Subramanyam *et al*., 2006; Williams *et al*., 2002). Another Hessian fly responsive gene, *Hfr-2*, encodes a chimeric protein with an entire amaranthin domain followed by a domain similar to haemolytic lectin/channel-forming toxins (Puthoff *et al*., 2005). The case for involvement of a set of lectins in Hessian fly resistance was strengthened by the observation that the sequence of *Hfr-3* is related to the WGA family of chitin-binding hevein domain lectins known to have a negative effect on insect growth and development (Eisemann *et al*., 1994; Murdock *et al*., 1990).

The effect of very low concentrations of WGA on the development of larvae from a variety of insect species demonstrates that this class of dietary lectins can act as powerful anti-nutrients. Murdock *et al*. (1990) screened 17 lectins in an artificial seed system to determine toxicity toward the cowpea weevil, and found that WGA caused the greatest delay in larval development and the highest larval mortality. In other larval feeding experiments, an artificial diet containing 0.3% WGA led to 50% weight reduction in Southern corn rootworm, while an artificial diet containing only 0.0059% WGA fed to European corn borer resulted in 50% larval mortality (Czapla and Lang, 1990). Unfortunately, Hessian fly larvae do not survive apart from the host plant, so feeding the WGA-like HFR-3 protein in an artificial diet was not possible.

Larval midguts contain several molecules that are potential targets for chitin-binding lectins with hevein domains, such as those encoded by the *WGA* genes and *Hfr-3*. Target molecule affinity is not limited to glycoconjugates of β-1,4-linked polymers of *N*-acetylglucosamine but may also include *N*-acetyl-D-neuraminic acid and other glycans (Van Damme *et al*., 1998). The epithelial surface of larval midguts is composed of glycosylated membrane proteins including hormone and growth factor receptors, and transport proteins (Pusztai and Bardocz, 1996). The PM that lines the larval midgut is rich in chitin that acts as a barrier for microbes and abrasive food found in the insect diet. Therefore, chitin-binding lectins could inhibit larval growth and development by binding to the PM and interfering with nutrient absorption, or

by disrupting the PM and allowing direct contact with microbes or plant substances that may damage the midgut (Harper *et al.*, 1998). In either case, the insect would be deterred from feeding or unable to assimilate nutrients and would eventually die of starvation.

Comparison of WGA amino acid sequences with the predicted sequence of HFR-3 protein revealed similarities and differences that may have a bearing on function. The predicted N-terminal signal peptide of HFR-3, although dissimilar in sequence, was similar in length to those known to be cleaved from WGA precursors during translation. Like the WGA proteins, the predicted HFR-3 protein contains four chitin-binding hevein domains with good conservation of the saccharide-binding amino acids at positions 19, 21, 23, 29 and 30 (Wright *et al.*, 1991). However, the *Hfr-3* sequence does not encode the 15-amino-acid vacuole-targeting region found adjacent to the fourth chitin-binding domain at the C-terminus of immature WGA proteins. Expression studies with a barley orthologue of WGA in transgenic tobacco show that deletion of this domain results in secretion of the encoded protein (Bednarek *et al.*, 1990). The *Hfr-3* mRNA sequence encodes only one amino acid after the final cysteine of the fourth chitin-binding domain and the first termination codon is followed just three bases later by a second termination codon. A software-based translation of the subsequent mRNA trailer sequence predicts a corresponding amino acid sequence that bears no similarity to the vacuole-targeting peptide of the WGA proteins. Because the HFR-3 protein lacks this domain, it may be secreted to the surface of the leaf sheath where the lapping mouthparts of first-instar Hessian fly larvae consume plant cell exudates.

Hfr-3 and its orthologues on the group 7 chromosomes of wheat constitute a previously unknown subfamily of the *WGA* genes that reside on the group 1 chromosomes (Etzler, 1985). Although the expression of *WGA* and *Hfr-3* mRNAs were similar in vegetative organs of uninfested plants (Fig. 4) as might be expected of genes derived from a common origin, only *Hfr-3* showed an induced response to Hessian fly larvae. WGA is primarily considered an embryo-specific protein; however, like HFR-3 its abundance also increases during plant defence (Raikhel *et al.*, 1984). Roots challenged with the fungal pathogen *Pythium ultimum* showed a fivefold increase in WGA content 48 h after infection, with a subsequent decrease as the protein was secreted from the roots into the surrounding medium. Despite similar roles in defence, *Hfr-3* may be more closely related to the gene in a wheat relative, *Agropyron repens* (Cammue *et al.*, 1985), which contains WGA-like leaf-specific lectins that are not identical to the WGA proteins in the embryos. The expression of *Hfr-3* further differed from *WGA* in that exogenous application of ABA did not lead to increased levels of *Hfr-3* mRNA, whereas WGA accumulates to higher levels after ABA treatment in organs where it is normally expressed (Mansfield and Raikhel, 1990; Raikhel *et al.*, 1993). These data suggest that even though *Hfr-3* and the *WGA* genes share significant sequence similarity, they may be activated through distinct signalling pathways.

Our data support an earlier study suggesting that the wheat resistance response is localized at the sites of avirulent larval attack rather than being systemic. During mixed infestations with both virulent and avirulent Hessian flies, virulent larvae that are feeding a few millimetres away from avirulent larvae developed normally (Grover *et al.*, 1989). In addition, avirulent larvae that hatched a few days before virulent larvae, and had triggered early stages of the resistance response, were able to survive once virulent larvae began to feed a few millimetres away (C.E. Williams *et al.* unpublished observations). This obviation of resistance occurs because virulent larvae alter the developmental fate of plant cells at the base of the leaf sheath such that nutritive tissue forms where the larvae are attempting to feed (Harris *et al.*, 2006). We suggest that avirulent larvae move away from the few cells that are producing resistance proteins, such as HFR-3, and begin feeding on adjacent nutritive tissue that delivers plant cell exudates to the surface of the leaf sheath. Consequently, wheat genes such as *Hfr-3*, putatively involved in defence against Hessian fly, would be expected to respond in only a few cells at sites probed by avirulent larvae. Our experiment with different levels of Hessian fly infestation supports a hypothesis of localized resistance. *Hfr-3* mRNA levels were 6.1-fold higher in plants infested with 21–30 avirulent larvae than in plants infested with one (Fig. 2c, 4 days after egg hatch), consistent with expression being influenced by the number of feeding sites.

The responsiveness of *Hfr-3* during compatible interactions with Hessian fly and the aphid *R. padi* suggests that in addition to its involvement in induced resistance, this gene plays a role in inducible basal defence against virulent sucking insects. However, the 36-fold increase in *Hfr-3* mRNA levels above controls achieved by 21–30 virulent Hessian fly larvae was lower than the increase (46-fold) caused by a single avirulent larva. The toxicity of dietary lectins has been shown to be dependent on the amount ingested. For example, WGA comprising 0.2% of the cowpea weevil artificial diet delayed larval development by 6.5 days whereas 1% WGA increased the delay to 22.8 days (Murdock *et al.*, 1990). Thus, survival of virulent Hessian fly larvae may result from the low concentration of HFR-3 protein produced in compatible interactions, coupled with the larva's ability to move out of localized areas producing the lectin, and other defence molecules, and into regions of developing nutritive tissue.

Accumulation of *Hfr-3* mRNA in the crown tissues where larvae are active suggests that both virulent and avirulent larvae ingest HFR-3 while attempting to feed on the plant. Although *Hfr-3* mRNA levels were 4–8 times higher in resistant and susceptible plants infested with 21–30 larvae than in plants infested with a single larva (Fig. 2c), the encoded protein was detectable only in resistant plants infested with avirulent larvae (incompatible interaction, Fig. 5a). However, the HFR-3 protein was detected in

both virulent and avirulent larvae that had fed on the plant, and was absent in larvae that had not yet fed and were emerged in water. These results indicated that the protein was ingested by the insects in both compatible and incompatible interactions (Fig. 5b).

Virulent larvae (compatible interactions) consume plant exudates and rapidly increase in size during the first instar. By contrast, avirulent larvae consume little (Shukle *et al*., 1992), do not increase in size and appear to die of starvation. Although the concentration of HFR-3 protein is low in plants during incompatible interactions, the large volume consumed by virulent larvae leads to detectable levels of HFR-3 within the larvae. Yet virulent larval enzymes that detoxify plant defence compounds and deal with stress appear to be more responsive in virulent than in avirulent larvae (Giovanini *et al*., 2006; Mittapalli *et al*., in press). During the first week of development, as virulent larvae move away from plant cells expressing defence molecules and into developing nutritive tissue that appears incapable of mounting a defence response, the levels of ingested HFR-3 would decrease. Only virulent larvae induce plant nutritive tissue. Consequently, each new site probed by avirulent larvae would lead to induction of defence molecules capable of adversely affecting the PM of the midgut or deterring feeding. We propose that avirulent larvae die of starvation via two processes: they are unable effectively to deal with induced plant defences such as toxic lectins, so they are deterred from feeding and thus attempt to inject eliciters into the plant less often; plus they are unable to alter the plant's developmental fate to produce nutritive tissue so that they can establish a permanent feeding site. When avirulent larvae coexist with virulent larvae, both are able to survive once nutritive tissue is established in the plant.

In summary, we have characterized a novel gene encoding a lectin with hevein domains that is associated with Hessian fly resistance in wheat. Our conclusions are based on the observed accumulation of *Hfr-3* mRNA and HFR-3 protein in wheat crown tissues where avirulent first-instar larvae attempt to establish feeding sites during the first 4 days of the incompatible interaction. The presence of HFR-3 in the larval diet during the first-instar may contribute to larval mortality that is characteristic of the incompatible interaction.

EXPERIMENTAL PROCEDURES

Cloning of *Hfr-3* (Fig. 1)

A GeneCalling experiment (Curagen Corp.) yielded a 132-bp fragment corresponding to an mRNA that was differentially expressed in *H13* resistance gene-containing wheat during compatible (challenged with a *vH9* Hessian fly stock) and incompatible (Biotype L Hessian fly stock) interactions. The GeneCalling technique is described in detail by Bruce *et al.* (2000). Briefly, cDNA derived from mRNA (from both compatible and incompatible interactions) was digested with restriction enzymes and ligated to adapters. The fragments were amplified by PCR using fluorescamine-tagged primers, which allowed the amplicons to be digitally quantified during electrophoresis. Fluorescence levels were compared for amplicons of identical size to identify expression differences between compatible and incompatible interactions. About 100 fragments were identified, sequenced and subjected to BLAST analysis.

The GeneCalling experiment yielded a fragment that corresponded to a WGA-like sequence. This sequence was used to design quantitative real-time PCR (qRT-PCR) primers (Primer Express, Applied Biosystems, Foster City, CA; primer sequence shown below) and differential mRNA levels were confirmed for *Hfr-3* (GenBank accession number DQ462308). A near full-length cDNA corresponding to this sequence was obtained using 5′ and 3′ RACE (GeneRacer Kit, Invitrogen, Carlsbad, CA; 5′ RACE gene-specific primer 5′-GCAGCCAGTATGGGTCTTGTGGTCTTGG-3′ and 3′ RACE gene-specific primer 5′-TCAGCCAGGAAGGAAGGAGTG-TACCACA-3′). After sequencing the two RACE products, new forward (5′-ATGAAGGGCCTCTTGCTGT-3′) and reverse (5′-CTAGTTGCATGCACCATTCTG-3′) primers were used to amplify through the *Hfr-3* ORF using high-fidelity DNA polymerase (Platinum *Pfx* DNA Polymerase, Invitrogen). The PCR cycling parameters were 94 °C for 2 min, 25 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 1 min, and a final extension step of 68 °C for 10 min. The PCR template was cDNA synthesized from mRNA isolated from the '*H9*-Iris' wheat line 3 days after hatch of Hessian fly Biotype L eggs (incompatible interaction). The resulting PCR fragment was gel-purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA), cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen) and sequenced by the Purdue University Genomics Core Facility. This *Hfr-3* clone (UPW1Hfr3) was used as template to amplify an *Hfr-3* ORF PCR fragment that was used as a template to synthesize ^{32}P-labelled *Hfr-3* probes for Northern and Southern hybridization experiments.

Hessian fly infestation and plant growth (all experiments)

Plants (10–12 per pot) were grown in 10-cm plastic pots filled with a peat and bark-based growing medium (Premier Horticulture Inc., Quakertown, PA) and covered with a 3-cm layer of vermiculite in a growth chamber maintained at 18 °C, 60% relative humidity and a 24-h photoperiod. Irradiance for all experiments was between 250 and 300 μmol/m^2/s. When plants reached the two-leaf stage, they were covered with vented plastic cups and infested with *vH9* (virulent to wheat containing the *H9* resistance gene) or Biotype L (avirulent on *H9*-Iris) adult Hessian flies (six females and two males per pot). Twenty-four hours after infestation, flies were removed by gently brushing the plants. Control and experimental plants were treated identically except that the pots containing control plants received no flies.

Following infestation, plants were selected at random each day and dissected to determine when first-instar Hessian fly larvae reached the base of the crown (aerial portion of a seedling immediately above the roots and below the ligule of the first leaf). For all experiments, the basal 1 cm of the crown, where Hessian fly larval attack is concentrated, was harvested from at least ten seedlings per time point directly into liquid nitrogen and stored at −80 °C until RNA isolation. To preserve the physical integrity and minimize wound response of wheat seedlings, Hessian fly larvae were not removed from plant tissue collected for experimentation. Sixteen days after infestation, all remaining plants were dissected and living larvae (compatible interaction) or dead larvae (incompatible interaction) were counted to determine infestation level and to verify that the correct interaction had taken place. Infestations of the wheat differential lines carrying resistance genes *H3*, *H5*, *H6* and *H7/H8* plus *H9*-Iris were included as controls in each experiment to verify the genotype of fly stocks. Biotype L survives on all differential line genotypes, but fails to develop on *H9*-Iris, whereas *vH9* flies survive on *H9*-Iris. Two sets of tissue (biological replicates) were grown for each experiment. The *H9*-Iris line of wheat (containing the *H9* Hessian fly resistance gene) was used in all experiments except for the experiment shown in Fig. 2b. Biotype L (avirulent on all plants in this study that contained resistance genes) and *vH9* (virulent on plants containing the *H9* resistance gene) Hessian flies were maintained as purified laboratory stocks at the USDA-ARS Crop Production and Pest Control Research Unit (West Lafayette, IN).

Tissue to quantify *Hfr-3* mRNA levels in response to Hessian fly larvae (Fig. 2b)

Wheat plants were grown as described above and infested at the two-leaf stage with Hessian fly Biotype L. Uninfested control plants were also grown. Infestation of the wheat line 'Blueboy', which carries no Hessian fly resistance genes, resulted in a compatible interaction. Six wheat lines, each carrying different Hessian fly resistance genes, were infested with Hessian fly Biotype L to yield six different incompatible interactions. Wheat lines containing Hessian fly resistance genes included '97211' (unmapped *R* gene), '97215' (unmapped *R* gene), 'Molly' (*H13 R* gene), '921676' (*H14 R* gene), '921682' (*H16 R* gene) and '921680' (*H17 R* gene). The *Hfr-3* primers, forward (5′-CACCATGCACCAACAACTATTGT-3′) and reverse (5′-CAGCCAGGAAGGAAGGAGTGT-3′), were used in this and all other qRT-PCR experiments.

Tissue to quantify *Hfr-3* mRNA in response to infestation levels (Fig. 2c)

H9-Iris plants were grown as described above and using three different fly infestation levels (one female with two males; three females with three males; and six females with four males). The adult flies were caged in a cup placed on the top of each pot and left for 2, 8 or 24 h to ensure variable numbers of larvae feeding at the crown tissue on different plants. All plants, including controls, were dissected before being frozen, and the tissues were pooled according to the number of larvae present at the crown of each plant. Tissues containing one larva and tissues containing 21–30 larvae were used for RNA isolation and quantification of *Hfr-3* mRNA levels.

Plant tissues for analysis of *Hfr-3* and *WGA* mRNA levels (Fig. 4)

Leaf blades, ligules, crowns and roots were harvested from two-leaf-stage *H9*-Iris plants grown in growth chambers as described above and in Puthoff *et al.* (2005), and stem tissue was harvested from fully headed *H9*-Iris plants that were grown under field conditions at the Purdue University Agronomy Center for Research and Education. Plants were not infested with insects. All tissue was harvested directly into liquid nitrogen.

qRT-PCR to quantify levels of *WGA* mRNA was set up using the forward (5′-ATCGCCACCAACTCCACTCT-3′) and reverse (5′-AAAACCGTACGTGGCAATGG-3′) primers designed from the *WGA-B* sequence (GenBank accession number J02961). The 101-bp *WGA-B* amplicon sequence has 87% identity with the corresponding sequence in the *WGA-D* sequence, 85% with *WGA-A* and no significant similarity with the *Hfr-3* sequence.

Aphid and virus treatments (Fig. 3)

H9-Iris wheat plants were grown as described above and in Puthoff *et al.* (2005), and were infested at the two-leaf stage with the bird cherry-oat aphid, *R. padi*, carrying either no virus or carrying two viruses: BYDV (PAV strain) and CYDV. Uninfested control plants were also grown. Aphids were introduced on to the soil below the plants. Infested pots were placed inside clear plastic bags, returned to the growth chamber and maintained for 4 h. All plants (including uninfested controls) were then treated with the insecticide Malathion to kill aphids, according to label directions, and plastic bags were removed. At each time point, the above-soil portion of ten seedlings was collected into liquid nitrogen and stored at −80 °C until RNA isolation. Aphids were provided by Dr J. A. Anderson (USDA-ARS Crop Production and Pest Control Research Unit, West Lafayette, IN).

Chewing insect and wounding treatments (data not shown)

H9-Iris wheat plants were grown as described above and in Puthoff *et al.* (2005), and at the two-leaf stage were infested with FAW larvae, *S. frugiperda*, wounded by pinching seedlings with forceps every 3 cm from soil level to the leaf tips, or received

no treatment (untreated controls). FAW larvae were applied at a rate of one larva per plant and only plants with visible FAW damage were harvested. At each time point, the above-soil portion of at least five seedlings was collected into liquid nitrogen and stored at −80 °C until RNA isolation. FAW were provided by Dr W. P. Williams (USDA-ARS Corn Host Plant Resistance Research Unit, MS).

Elicitor molecule treatment (data not shown)

H9-Iris wheat plants were grown as described above, and at the two-leaf stage were sprayed to run-off (approximately 2 mL per pot) with aqueous solutions of ABA (100 μM), MeJA (45 μM) or SA (10 mM). Control plants were sprayed with water only. Time points for tissue collection were zero days (immediately prior to treatment), and 1, 2, 3 and 4 days after treatment. At each time point, complete aerial tissue including the crown and leaf blades of 40–55 seedlings were collected into liquid nitrogen and stored at −80 °C until RNA isolation.

RNA isolation and cDNA synthesis (Figs 2–4)

Total RNA was isolated using TRIzol reagent (Invitrogen) according to methods described by Puthoff *et al.* (2005). cDNA synthesis was carried out as described in Puthoff *et al.* (2005) using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) with the following modifications. Total RNA (4.35 μg RNA in 10 μL water) was treated using the DNA-free Kit (Ambion, Austin, TX) to remove contaminating DNA. Ten microlitres of DNA-free RNA (containing 3 μg total RNA) was combined with 1 μL dNTP and 1 μL oligo d(T), heated for 5 min at 65 °C, then immediately placed on ice. To this cooled RNA, 2 μL of 10× buffer, 2 μL of 50 mM $MgCl_2$, 2 μL of 0.1 M DTT, 1 μL of RNaseOut and 1 μL of SuperScript II were added. Five microlitres of each reverse transcription reaction was removed and combined with 1 μL (1 : 5 diluted with water) ^{32}P-dCTP (Amersham, Piscataway, NJ) to serve as a tracer reaction (Puthoff *et al.*, 2003). All reactions were incubated at 42 °C for 2 h. Five microlitres of each tracer reaction was pipetted on to DE-81 filters (Fisher Scientific, Pittsburgh, PA) and allowed to air-dry. After washing (four times for 4 min each) in phosphate buffer (0.25 M each of Na_2HPO_4 and NaH_2PO_4), the filters were washed in water, followed by 95% EtOH, and allowed to air-dry. Filters were placed individually into 5 mL of scintillation solution (ScintiVerse, Fisher Scientific) and radioactivity was quantified by liquid scintillation counting. Counts measured for each tracer reaction were used to normalize the amount of cDNA per sample to 10 ng/μL for qRT-PCR amplifications. Normalization of cDNA among samples was verified by qRT-PCR using forward (5′-GGTGTCTCCGGTATCCTCCAA-3′) and reverse (5′-TGCTCCACACCAGCAGAAGT-3′) wheat ubiquitin primers (data not shown) designed from GenBank accession number X56803.

Quantitative real-time PCR (Figs 2–4)

Gene expression analysis by qRT-PCR was conducted as described in Puthoff *et al.* (2005) and Giovanini *et al.* (2006). Two microlitres of normalized cDNA (20 ng) was combined with 10 μL SYBR Green Master Mix (Applied Biosystems), 0.5 μL of each primer (10 μM) and 7 μL of water per reaction. qRT-PCR was carried out on an ABI Prism 7000 (Applied Biosystems) with the following cycling parameters: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Following qRT-PCR, a melt curve analysis was used to check primer specificity and confirm the presence of a single PCR product. Relative mRNA levels were calculated using the standard curve method (*User Bulletin 2: ABI PRISM 7700 Sequence Detection System*). Briefly, a standard curve was generated from six serially diluted cDNA samples (the undiluted sample was a pool of equal aliquots from all experimental samples). Each time a qRT-PCR amplification was performed, the pooled, serially diluted cDNA samples were included in the same 96-well plate as the experimental samples. The threshold cycle (Ct) for each dilution was plotted against its cDNA concentration (with an arbitrary starting quantity of 1 for the undiluted pooled sample) and a regression equation was used to generate arbitrary expression values (AEVs) for the experimental samples. For gene expression analysis by qRT-PCR, the data shown in the figures represent plants collected from two biological replicate experiments, with each biological replicate subjected twice to qRT-PCR analysis (i.e. two technical replicates).

Statistical analysis of qRT-PCR data (Figs 2 and 3)

Statistical significance of the data was determined by ANOVA using the PROC MIXED procedure of SAS (SAS Institute, 1999). The analysis model included treatments (compatible, incompatible, uninfested), time points, and interaction between treatments and time points as fixed effects. Data from two biological replicates, each composed of two technical replicates, were combined and included as a random effect in the analysis model. Orthogonal contrasts were used to evaluate whether the effects of treatment and/or treatment-by-time-point interaction were significant. The *P*-values from the ANOVA test are indicated in the results only for time points of interest. Two treatments were considered to have statistically different fold changes at a given time point if the *P*-value associated with the contrast was < 0.05. Fold changes were calculated from AEVs.

Northern hybridization (Fig. 2d)

Total RNA was isolated from uninfested, *vH9*-infested (compatible interaction), and Hessian fly Biotype L-infested *H9*-Iris plants (incompatible interaction) using the methods described above.

For each sample, total RNA (12 μg RNA in 10 μL water) was combined with 10 μL of denaturing RNA loading buffer (547 μL formamide, 182.5 μL formaldehyde, 200 μL 10× MOPS, 66 μL glycerol, and a few grains of bromphenol blue) and 1 μL of ethidium bromide solution (0.4 mg/mL). RNA samples were heated at 65 °C for 15 min with vigorous vortexing before and after incubation. Total RNA was separated on a 1.2% agarose gel containing 5% formaldehyde and run for 3 h at 72 V in 1× MOPS buffer. Following electrophoresis, RNA was transferred to Hybond-XL membrane (Amersham) and cross-linked to the membrane at 12 000 μJ/cm^2 using a UV cross-linker (UVC 500, Hoefer, San Francisco, CA). Membrane-bound RNA was hybridized with a ^{32}P-labelled *Hfr-3* probe. The UPW1Hfr3 clone served as a template for generating an amplicon covering the entire ORF, and that amplicon served as template for a random priming/labelling reaction (Prime-It II Random Primer Labeling Kit, Stratagene, La Jolla, CA) to generate the probe for hybridization. Prehybridization, hybridization and wash steps were carried out using PerfectHyb Plus Hybridization Buffer (Sigma, St Louis, MO) according to the manufacturer's instructions. Blots were exposed to Biomax MR film (Eastman Kodak Co., Rochester, NY) with Biotech L-Plus intensifying screens (Fisher Scientific, Pittsburg, PA) for 5 days at −80 °C.

Western immunodetection of HFR-3 protein (Fig. 5)

Infested *H9*-Iris plants were immersed in ddH_2O in a Petri dish and carefully dissected to expose the larvae. Gentle agitation of seedlings in the water caused larvae to be washed out so that both plant and insect samples could be collected. Water containing larvae was pipetted into 1.5-mL tubes and the larvae settled to the bottom of the tubes after several minutes. The water was carefully removed and larvae were washed with ddH_2O inside the tubes. Washed larvae were allowed to settle again. The water was removed and tubes containing only larvae were frozen in liquid nitrogen and stored at −80 °C until protein isolation. Uninfested control plants were dissected in the same manner as Hessian-fly-infested plants. Crown tissues were removed from seedlings as described above and stored at −80 °C until use.

Approximately 100 mg of frozen *H9*-Iris crown tissues was homogenized in a tube with 300 μL of extraction buffer (0.125 M Tris-HCl, pH 6.8, 1% SDS) and 3 μL Protease Inhibitor Cocktail for plant cell and tissue extracts (Sigma). Samples were vortexed, boiled for 5 min and centrifuged at 10 000 ***g*** for 5 min at 4 °C. The supernatant was transferred to a new tube and centrifuged again at 10 000 ***g*** for 5 min at 4 °C to remove debris. The upper debris-free layer was transferred to a new tube and the concentration of total proteins was quantified using the Bradford Method Protein Assay Kit (Amresco, Solon, OH). For each sample, 20 μg of protein was mixed with an equal volume of 2× Laemmli Sample Buffer (Bio-Rad, Hercules, CA) containing 5% (v/v) β-mercaptoethanol. Samples were boiled for 5 min, and then loaded on to 4–10% Tris-HCl Ready Gels (Bio-Rad). SDS-PAGE was carried out in a Mini-PROTEAN 3 apparatus (Bio-Rad) at 130 V for 1 h with 1× Tris/glycine/SDS running buffer (Bio-Rad). Following electrophoresis, proteins were transferred to Immuno-Blot PVDF membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 100 V for 1 h. Antiserum raised against a synthetic HFR-3 peptide (WADLKCGHLDNGKL; Zymed Laboratories, San Francisco, CA) diluted 1 : 5000 (0.1 μg/mL) was used as the primary antibody, and peroxidase-conjugated anti-rabbit IgG (Sigma) diluted 1 : 10 000 was used as the secondary antibody. Western immunodetection was carried out using SuperBlock T20 (TBS) Blocking Buffer (Pierce, Rockford, IL) and SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's protocol. Detection was achieved by exposing membranes to film for 1–3 min. Background noise on the membrane was reduced by (Fig. 5b only) using Erase-It Background Eliminator Kit (Pierce).

Southern hybridization (Fig. 6)

Southern hybridization experiments were conducted to determine chromosomal localizations of the *Hfr*-3-like genomic sequences. Genomic DNA was isolated from *H9*-Iris, 'Chinese Spring' wheat, and Chinese Spring-derived nullitetrasomic deletion stocks (Sears, 1966) using the CTAB method (Richards *et al.*, 1994). DNA samples (20 μg) were digested with *Eco*RV (Invitrogen), which does not cut within the *Hfr-3* genomic sequence, separated on a 0.8% agarose gel in 1× TAE buffer at 40 V for 6 h and transferred to Hybond-XL membrane (Amersham). Membrane-bound DNA was hybridized with a ^{32}P-labelled *Hfr-3* probe, synthesized using an *Hfr-3* ORF PCR fragment as template as described for the Northern hybridizations above. Prehybridization, hybridization and wash steps were carried out using PerfectHyb Plus Hybridization Buffer (Sigma) according to the manufacturer's instructions. Blots were exposed to Biomax MR film (Eastman Kodak) with Biotech L-Plus intensifying screens (Fisher Scientific) for 14 days at −80 °C.

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REFERENCES

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.

Bednarek, S.Y., Wilkins, T.A., Dombrowski, J.E. and Raikhel, N.V. (1990) A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco. *Plant Cell*, **2**, 1145–1155.

Bruce, W., Folkerts, O., Garnaat, C., Crasta, O., Roth, B. and Bowen, B. (2000) Expression profiling of the Maize flavonoid pathway genes controlled by estradiol-inducible transcription factors CRC and P. *Plant Cell*, **12**, 65–79.

Cammue, B., Stinissen, H.M. and Peumans, W.J. (1985) A new type of cereal lectin from leaves of couch grass (*Agropyrum repens*). *Eur. J. Biochem.* **148**, 315–322.

Cartwright, W.B., Caldwell, R.M. and Compton, L.E. (1959) Responses of resistant and susceptible wheats to Hessian fly attack. *Agron. J.* **51**, 529–531.

Chrispeels, M.J. and Raikhel, N.V. (1991) Lectins, lectin genes, and their role in plant defense. *Plant Cell*, **3**, 1–9.

Czapla, T.H. and Lang, B.A. (1990) Effect of plant lectins on the larval development of European corn borer (Lepidoptera: Pyralidae) and Southern corn rootworm (Coloeptera: Chrysomelidae). *J. Econ. Entomol.* **83**, 2480–2485.

Eisemann, C.H., Donaldson, R.A., Pearson, R.D., Cadogan, L.C., Vuocolo, T. and Tellam, R.L. (1994) Larvicidal activity of lectins on *Lucilia cuprina*: mechanism of action. *Entomol. Exp. Appl.* **72**, 1–10.

Etzler, M.E. (1985) Plant lectins: molecular and biological aspects. *Ann. Rev. Plant Physiol.* **36**, 209–234.

Flor, H.H. (1955) Host–parasite interaction in flax rust—its genetics and other implications. *Phytopathology*, **45**, 680–685.

Gallun, R.L. (1977) Genetic basis of Hessian fly epidemics. *Ann. NY Acad. Sci.* **287**, 223–229.

Gallun, R.L. (1978) Genetics of biotypes B and C of the Hessian fly. *Ann. Entomol. Soc. Am.* **71**, 481–486.

Gattiker, A., Gasteiger, E. and Bairoch, A. (2002) ScanProsite: a reference implementation of a PROSITE scanning tool. *Appl. Bioinformatics*, **1**, 107–108.

Giovanini, M.P., Puthoff, D.P., Nemacheck, J.A., Mittapalli, O., Saltzmann, K.D., Ohm, H.W., Shukle, R.H. and Williams, C.E. (2006) Gene-for-gene defense of wheat against the Hessian fly lacks a classical oxidative burst. *Mol. Plant–Microbe Interact.* **191**, 1023–1033.

Grover, P.B. Jr (1995) Hypersensitive response of wheat to the Hessian fly. *Entomol. Exp. Appl.* **74**, 283–294.

Grover, P.B., Jr, Shukle, R.H. and Foster, J.E. (1989) Interactions of Hessian fly (Diptera: Cecidomyiidae) biotypes on resistant wheat. *Environ. Entomol.* **18**, 687–690.

Harper, M.S., Hopkins, T.L. and Czapla, T.H. (1998) Effect of wheat germ agglutinin on formation and structure of the peritrophic membrane in European corn borer (*Ostrinia nubilalis*) larvae. *Tissue Cell*, **30**, 166–176.

Harris, M.O., Freeman, T.P., Rohfritsch, O., Anderson, K.G., Payne, S.A. and Moore, J.A. (2006) Virulent Hessian fly (Diptera: Cecidomyiidae) larvae induce a nutritive tissue during compatible interactions with wheat. *Ann. Entomol. Soc. Am.* **99**, 305–316.

Hatchett, J.H. and Gallun, R.L. (1970) Genetics of the ability of the Hessian fly, *Mayetiola destructor*, to survive on wheats having different genes for resistance. *Ann. Entomol. Soc. Am.* **63**, 1400–1407.

Mansfield, M.A. and Raikhel, N.V. (1990) Abscisic acid enhances the transcription of wheat-germ agglutinin mRNA without altering its tissue-specific expression. *Planta*, **180**, 548–554.

Mittapalli, O., Shukle, R.H., Sardesai, N., Giovanini, M.P., Williams, C. and E. (in press) Expression patterns of antibacterial genes in the Hessian fly. *J. Insect Physiol.* in press.

Murdock, L.L., Huesing, J.E., Nielsen, S.S., Pratt, R.C. and Shade, R.E. (1990) Biological effects of plant lectins on the cowpea weevil. *Phytochemistry*, **29**, 85–89.

Murdock, L.L. and Shade, R.E. (2002) Lectins and proteases inhibitors as plant defenses against insects. *J. Agric. Food Chem.* **50**, 6605–6611.

Peumans, W.J. and Van Damme, E.J.M. (1995) Lectins as plant defense proteins. *Plant Physiol.* **109**, 347–352.

Pusztai, A. and Bardocz, S. (1996) Biological effects of plant lectins on the gastrointestinal tract: metabolic consequences and applications. *Trends Glycosci. Glycotechnol.* **8**, 149–165.

Puthoff, D.P., Nettleton, D., Rodermel, S.R. and Baum, T.J. (2003) Arabidopsis gene expression changes during cyst nematode parasitism revealed by statistical analyses of microarray expression profiles. *Plant J.* **33**, 911–921.

Puthoff, D.P., Sardesai, N., Subramanyam, S., Nemacheck, J.A. and Williams, C.E. (2005) *Hfr-2*, a wheat cytolytic toxin-like gene, is up-regulated by virulent Hessian fly larval feeding. *Mol. Plant Pathol.* **6**, 411–423.

Raikhel, N.V., Lee, H.-I. and Broekaert, W.F. (1993) Structure and function of chitin-binding proteins. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 591–615.

Raikhel, N.V., Mishkind, M.L. and Palevitz, B.A. (1984) Characterization of a wheat germ agglutinin-like lectin from adult wheat plants. *Planta*, **162**, 55–61.

Raikhel, N.V. and Wilkins, T.A. (1987) Isolation and characterization of a cDNA clone encoding wheat germ agglutinin. *Proc. Natl Acad. Sci. USA*, **84**, 6745–6749.

Richards, E., Reichardt, M. and Rogers, S.J. (1994) Preparation of plant DNA using CTAB. Unit 2.3.3–2.3.5. Supplement 27. In: *Current Protocols in Molecular-Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., eds). John Wiley & Sons: Hoboken NJ.

Sardesai, N., Subramanyam, S., Nemacheck, J. and Williams, C. (2005) Modulation of defense-response gene expression in wheat during Hessian fly larval feeding. *J. Plant Interact.* **1**, 39–50.

SAS Institute. (1999) *SAS/STAT® User's Guide*, Version 8. Cary, NC: SAS Institute.

Sears, E.R. (1966) Nullisomic-tetrasomic combinations in hexaploid wheat. In: *Chromosome Manipulations and Plant Genetics* (Lewis, R.R. and Lewis, K.R., eds), pp. 29–45. Edinburgh: Oliver and Boyd.

Shukle, R.H., Grover, P.B. Jr and Foster, J.E. (1990) Feeding of Hessian fly (Diptera: Cecidomyiidae) larvae on resistant and susceptible wheat. *Envirion. Entomol.* **19**, 494–500.

Shukle, R.H., Grover, P.B. Jr and Mocelin, G. (1992) Responses of susceptible and resistant wheat associated with Hessin fly (Diptera: Cecidomyiidae) infestation. *Envirion. Entomol.* **21**, 845–853.

Smith, J.J. and Raikhel, N.V. (1989) Nucleotide sequences of cDNA clones encoding wheat germ agglutinin isolectins A and D. *Plant Mol. Biol.* **13**, 601–603.

Subramanyam, S., Sardesai, N., Puthoff, D.P., Meyer, J.M., Nemacheck, J.A., Gonzalo, M. and Williams, C.E. (2006) Expression of two wheat defense-response genes, *Hfr-1* and *Wci-1*, under biotic and abiotic stresses. *Plant Sci.* **170**, 90–103.

Van Damme, E.J.M., Peumans, W.J., Barre, A. and Rougé, P. (1998) Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. *Crit. Rev. Plant Sci.* **17**, 575–692.

Williams, C.E., Collier, C.C., Nemacheck, J.A., Chengzhi, L. and Cambron, S.E. (2002) A lectin-like wheat gene responds systemically to attempted feeding by avirulent first-instar Hessian fly larvae. *J. Chem. Ecol.* **28**, 1411–1428.

Wright, H.T., Sandrasegaram, G. and Wright, C.S. (1991) Evolution of a family of *N*-acetylglucosamine binding proteins containing the disulfide-rich domain of wheat germ agglutinin. *J. Mol. Evol.* **33**, 283–294.